NUTRITION AND PHYSIOLOGY OF ENDAMOEBA HISTOLYTICA

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CONTENTS

I. INTRODUCTION

Amebiasis has become a disease of major concern in the United States although until recently, physicians believed that its incidence was insignificant in this country, being primarily limited to the tropics. Little information is available on the biochemistry and physiology of *Endamoeba histolytica*, the etiological agent of amebiasis. Research on the nonclinical aspects of *E. histolytica* has lagged behind the therapeutic studies, and the information available on this protozoan organism is pitifully limited as compared with the knowledge on pathogenic bacteria. In view of the recent trends towards studying the physiology and metabolism of a disease producing organism as a means of providing a rational therapy, more and more interest is presently being devoted to the physiology of the amebas causing amebiasis.

The literature on *E. histolytica* is scattered widely among medical journals and journals of parasitology. Publications on the clinical aspects as well as the therapeutic aspects of amebiasis are numerous, but physiological studies have been neglected. The purpose of this review is to summarize and analyze the information available on the physiology of *E. histolytica*.

[1] The author is indebted to Dr. Erich Hirschberg for reading the manuscript and making numerous valuable suggestions.

II. NUTRITIONAL REQUIREMENTS

Of prime importance in studying the physiological aspects of any organism is the study of its nutritional requirements. The nutritional requirements of *E. histolytica* are quite different from those of other protozoa which have been studied, and little is known about the nutrition of *E. histolytica* as compared with that of other parasitic protozoa.

1. *Culture media.* As early as 1916, Penfold *et al.* (72) claimed to have cultivated *E. histolytica* in a medium containing nutrient broth and a pancreatic digest preparation. Yoshida (109) reported that the amebas lived for 36 to 72 hours *in vitro* in a medium consisting of one part horse serum, four parts Ringer's solution, and red blood cells. Cutler (28) also claimed to have cultivated the amebas, but as the results of these early workers were not confirmed by protozoologists, the credit for the first successful cultivation of the amebas is usually given to Boeck and Drbohlav (9, 10) who cultivated the amebas without great difficulty upon special media. Their culture method was demonstrated before the American Society of Tropical Medicine in 1924 in Chicago and also before the National Academy of Sciences in the same year at Boston. Their medium became known as the Locke-egg-serum (L.E.S.) medium. Since this was the first successful cultivation of *E. histolytica*, a considerable number of studies were performed on the cultural behavior of the amebas. Boeck and Drbohlav (9, 10) maintained the culture by subculturing every 48 hours; the usual method of making subcultures consisted of withdrawing a few drops of the sediment at the bottom of the culture tube by means of a sterile capillary pipette with a rubber nipple attached and inoculating new tubes of the medium.

After the publications of Boeck and Drbohlav appeared, several modifications of their medium became known. Craig (23, 24) simplified the medium by eliminating the coagulated egg slant; his was a liquid medium containing only Locke's solution and serum. Later, Craig and St. John (25) stated that none of the chemicals in Locke's solution was necessary and that growth would occur in a medium consisting only of normal saline and inactivated serum with the addition of sodium chloride. It seems unlikely, however, that these studies were based on quantitative determinations. Occasionally, survival of amebas over a prolonged period of time is confused with growth. Drbohlav (32) suggested using blood agar covered with Ringer—egg white which was buffered and adjusted to a pH of 7.4.

In England, Dobell and Laidlaw (30, 31) also modified the Boeck-Drbohlav medium. They added starch to the cultures, stating that starch particles can be ingested by the amebas, thus supplying an abundant carbohydrate source (the role of starch is discussed in section II, part 2). Asparagine, as well as starch, was added to the culture media by Tanabe and Chiba (102, 103, 104).

In view of the finding that the amebas grew in the liver of an amebic patient, Cleveland and Collier (22) used a liver infusion agar slant covered with serum and saline. Growth of the amebas was satisfactory. Tsuchiya (105) prepared a simple medium consisting of sterile nutrient broth and a mixture of starch and charcoal. Charcoal was used to adsorb ammonia and hydrogen sulfide present

in the culture, thus reducing their possible deleterious effects upon the amebas. The use of charcoal on this basis might be questioned since no information was available then as to the gas production by the bacteria accompanying the amebas. Tsuchiya (105) further stated that this medium was specific for *E. histolytica* and that other *Endamoeba* species showed poor growth or none at all. St. John (89) prepared a medium containing extracted powdered heart muscle in a modified Locke's solution. A modification of the Cleveland and Collier medium was prepared by Craig (26).

Thus far, most of the modifications of the original Boeck-Drbohlav medium have consisted mainly of changes in the solid portion of the medium. The liquid portion of the medium has usually consisted of dilute preparations of human serum, animal serum, or egg albumin.

Lilly liver extract no. 343 was substituted for serum by Frye and Meleney (37). Some of the advantages of using liver extract, according to these investigators, were that the media could be resterilized several times without injury, that it was inexpensive, that it was available as a commercial preparation, that it was easily prepared for use, and that it required much less aseptic manipulation than serum. It is difficult to understand why the earlier workers did not go to the liver for nutritional factors in the cultivation of *E. histolytica* since it was known that amebas grew abundantly in this organ.

Producing large numbers of amebas in test tube cultures is expensive and time consuming. Frye and Meleney (36) grew amebas in large numbers in erlenmeyer flasks. With their method, one 250 ml flask containing a fluid overlay of 75 ml produced about the same number of amebas as 25 to 30 test tube cultures of 25 ml capacity. They reported that the amebas multiplied only at the bottom of the slant in test tube cultures, whereas in a flask they had the whole surface at the bottom of the flask upon which to multiply. Although this is a convenient method of obtaining a large pool of amebas for either metabolic or physiologic studies, cultures of amebas in flasks, however, are not as reliable as test tube cultures (59). The fluctuation in populations is much more variable and a longer incubation period is necessary to obtain the maximum population.

There is a constant search for a simpler medium which requires fewer manipulations and yet maintains the amebas for a long period of time. El Kordy (33) showed that hydatid fluid was an excellent medium for the cultivation of *E. histolytica*; he also cultivated the amebas in a medium made of an extract of dried hydatid scolices in Ringer's solution (43), as well as in fresh tomato juice (3). Inoki and his collaborators (51) cultivated the amebas in medium containing whole blood instead of serum. Most of the media of *E. histolytica* developed to this point were elaborated by blind substitutions of components which might stimulate or maintain the growth of amebas rather than the result of a rational approach to the cultural requirements of the amebas. Possibly a tabulation of all the common nutritional elements used in the various media would have focussed attention on the components that were needed for the cultivation of the amebas. Instead, the trend was to develop a medium containing different ingredients not used in other media.

Balamuth and Sandza (5) prepared a standardized fluid culture medium from an infusion of coagulated egg yolk made up in a buffered salt solution. This medium was almost transparent and wholly liquid; when Wilson's liver concentrate powder was added, the growth of the amebas was accelerated (4). The advantages of this first liquid medium over a diphasic medium are obvious. The transparency of this medium was also an advantage in that contamination of the media could be detected by the appearance of turbidity. Balamuth's medium was modified by Hitchcock and Rawson (49) who replaced the fresh egg yolk with Difco Bacto dehydrated coagulated egg yolk. These workers believed that this was a more standardized preparation and that the Bacto coagulated egg yolk would remain stable for several years. A medium which consisted of commercial dried egg yolk, yeast extract, and sodium chloride has also been described (47). The use of commercial preparations in making media is desirable since the media are less subject to variations. It now is evident that dried, coagulated egg yolk, in contrast to fresh eggs used by Boeck and Drbohlav, is satisfactory in preparing the media for *E. histolytica*.

Nelson (68) cultivated the amebas in a medium containing alcoholic extracts of liver preparations. Extracts from the liver of human, calf, beef, guinea pig, and cat were all effective in promoting growth of the amebas. Extracting substances from materials which have been shown to be effective in cultivating the amebas was designed to simplify the medium and also to concentrate the substance(s) required by the amebas.

All the media which were used contained bacteria accompanying the amebas. It became an accepted fact that the amebas required the bacteria and that if the bacteria were eliminated the amebas would not survive (the role of the bacteria is discussed in section V). However, Jacobs (54) grew the amebas in a medium in which bacteria were not obviously evident. The medium containing the usual egg slant-Locke's solution had been conditioned by the cultivation of *Escherichia coli* in it for 24 hours, after which the bacteria were killed by heat. After heating, rice powder and additional heat killed bacteria suspended in Locke's solution were added. This medium was inoculated with a culture of *E. histolytica* growing in association with *Clostridium perfringens*; penicillin was added to the culture tube to inhibit the latter. Thus, a relatively bacteria-free culture of amebas was maintained in this medium for a period of several months. Jacobs admitted, however, that there was no positive proof that bacteria were completely absent. Since the cultures were maintained for a number of months, multiplication of the amebas occurred. Jacobs found that it was very difficult to initiate this type of culture, possibly because the amount of nutritional material present in killed bacterial cells was inadequate to propagate the amebas. Further study of this interesting lead is definitely recommended.

Shaffer and his co-workers (93) cultivated *E. histolytica* with a 24 hour culture of *Streptobacillus* sp., an unidentified bacterium, in a fluid thioglycolate, glucose, and rice flour medium plus normal horse serum as a supplement. The growth of the bacteria was inhibited by the addition of penicillin; they claimed to have maintained this culture of amebas with few or no actively multiplying

bacteria for over one hundred transplants (92, 94, 95). We cannot accept a culture as being bacteria-free, however, if even a few bacteria are present in ameba cultures since small numbers of bacteria may carry on enough metabolism to furnish the amebas with the necessary nutrients.

The term synthetic medium is often abused in the study of protozoan cultures; all the media already described were made of very complex substances. Hansen and Anderson (46) described an essentially synthetic liquid medium for *E. histolytica* that was particularly successful in the cultivation of the amebas associated with organism *t*, another unidentified bacterium. This medium consisted of buffered saline solution containing trace minerals, 12 amino acids, 10 synthetic vitamins of the B complex, nucleic acid, cholesterol, and rice starch. These workers found that the liver extract previously used by others could be replaced by the synthetic vitamins (1). Hallman, Michaelson, and DeLamater (44) modified the medium of Hansen and Anderson by increasing the amino acids from 12 to 20. They also increased the amounts of tryptophan, cholesterol, magnesium, calcium, and nucleic acid and added a trace of glucose and glycogen to the medium. Rice starch was eliminated, and purified human plasma albumin and human serum mucoprotein were added. Hansen (45) prepared a medium, used for the routine propagation of stock cultures, that was a liquid type, easy to prepare and could be autoclaved. It contained proteose-peptone, Wilson's liver fraction L, cysteine, methionine, cholesterol, and a few inorganic salts. Whether or not these so-called "synthetic" media provided adequate nutrition for *E. histolytica* directly is to be questioned since a medium which is highly satisfactory for the associated bacteria will cause an indirect increased growth of the amebas.

Phillips and Rees (73, 75) first eliminated the bacteria from ameba cultures replacing them with *Trypanosoma cruzi*. The medium itself was very complex, containing agar, trypticase, defibrinated rabbit's blood, and the trypanosomes. The advantage of having a protozoan for an associate in place of bacteria is not clear. The complexity of the association still left much to be known regarding the role of the associated organism. The amebas grew with *T. cruzi* even when the trypanosomes were exposed to heat for 10 minutes at 48 C (73, 75), a treatment that apparently killed the trypanosomes. Nakamura and Anderson (65) found, however, that these heat treated trypanosomes still respired. Trypanosomes kept at 48 C for 10 minutes, 50 C for 10 minutes, or 54 C for 10 minutes were apparently dead; motility was absent, and growth did not occur when subcultured onto appropriate media. The amebas may be cultivated in a medium containing trypanosomes exposed to 48 C for 10 minutes but not in a medium containing trypanosomes exposed to 50 C for 10 minutes. Manometric studies indicated that trypanosomes kept at 48 C for 10 minutes still respired as measured by oxygen uptake, but those kept at 50 C for 10 minutes did not. A difference of 2 C proved to be determining between the ability or inability of the organism to multiply and to respire.

2. *Starch.* When Boeck and Drbohlav (9, 10) first cultivated *E. histolytica*, starch was not included as a part of the medium. As already noted, Dobell and

Laidlaw (30, 31) first introduced starch into the ameba cultures, stating that starch particles could be ingested by the amebas as a carbohydrate source. They also stated that the amebas lived longer in Boeck-Drbohlav medium with added starch than in the original medium containing glucose as the latter can be fermented readily by the bacteria, producing acid which made the medium unsuitable for amebic growth. These workers (31) studied 12 natural starches and found rice starch to be the best. An interesting observation made by them was that a strain of *E. histolytica* lost its pathogenicity when cultivated in Boeck-Drbohlav medium enriched with rice starch. Rees (79) found that the amebas grew better and more rapidly in media containing starch although the amebas became sluggish. He believed that the starch was not needed since the organisms grew well without it.

The exact role the starch plays in the nutrition of *E. histolytica* is not clear. Not only is there a controversy as to the requirements for starch, but if starch is required, its exact function is unknown. Most workers in the field believe that for optimum cultivation of the amebas starch is definitely required. Hansen and Anderson (46) established that rice starch could not be eliminated from a culture of *E. histolytica* in association with *t* grown in a partly synthetic medium since when they replaced starch with other carbohydrates, excess acidity resulted from fermentation by organism *t*. They suggested that the multiplication of amebas was directly related to the ingestion of rice granules, and also that starch served not only as a source of carbohydrate but also as an adsorbent for other ingredients in the fluid medium. With this in mind, Anderson and Hansen (1) made substitutions for starch using particles of charcoal, Fuller's earth, filtercel, celluflour, and barium sulfate but with no success. These workers studied the role of starch quite extensively and learned that the associate organism *t* does not require this polysaccharide. Clumps of starch granules 20 microns or more in diameter became etched and disintegrated into component granules 5 to 6 microns in diameter, which were then ingested by the amebas. Disintegration took place only in the presence of the protozoan and was not affected by organism *t* alone.

Another team of workers (44) showed that proteins of different structure and size could be used in place of rice powder when added to a synthetic base. Ovalbumin with a molecular weight of the order of 40,000 was the smallest protein successfully used. Gelatin, peptones, and peptides appeared to be inadequate substitutes for the starch, which suggested that molecular size and complexity of these substances might be an important factor in the metabolism of the amebas. Two schools of thought exist on the role of starch in the nutrition of *E. histolytica*; one holds that starch is an important carbohydrate source, and the other believes that it is primarily a source of protein since proteins extracted from rice powder can be substituted for rice powder in ameba cultures (44). Some workers even state that starch is important in supplying glucose to the amebas (95).

Hopkins and Warner (50) observed the amebas feeding upon rice starch grains, bacteria, fat globules, and yeast. They claimed to have followed rice

starch grains from the outside through ingestion, complete digestion, and egestion as fecal matter. They observed that the amebas had considerable power of selection of food since they contacted some starch grains without ingesting them, but engulfed others. Balamuth and Howard (6) showed that rice starch had a specific growth promoting influence on amebas in late stages of cultivation, even though bacterial growth continued to decline.

The optimum concentration of starch needed by the ameba-*Streptobacillus* sp. culture was 1 % by weight in 50 ml of the medium (93). An increase or decrease in rice flour resulted in diminished growth. Shaffer *et al.* (93) postulated that rice flour contained a factor which inhibited multiplication of the amebas, and also that bacteria acted in some manner on the rice flour which was beneficial or essential to the multiplication of the amebas. Others (100) have also shown a minimum, optimum, and maximum amount of rice powder for growth of amebas.

With our present knowledge of the nutritional status of *E. histolytica* it is difficult to define exactly the role of starch. Nevertheless, it has not been neglected in the nutritional studies of the amebas since most of the media developed since 1926 contain starch (4, 22, 30, 31, 33, 45, 46, 49, 102). In cultures of amebas growing with *T. cruzi*, however, starch is not required. Whether the trypanosomes furnish polysaccharide material found in starch or whether amebas growing with trypanosomes undergo a different type of metabolism is not known.

3. *Growth factors and growth stimulants.* The difficulty, is obvious, of establishing growth factors for an organism that requires the presence of a second organism whether it is a bacterium or another protozoan. The complexity of the growth requirements is such that it is difficult to establish any single growth factor for *E. histolytica*. Blood, serum, egg, liver, liver extract, peptone, rice powder, and a variety of complex materials have been used for the cultivation of *E. histolytica*, and apparently these constituents possess some growth factor or growth stimulant for the amebas. To establish or to identify the component is no easy task. Since bacteria are practically essential for the cultivation of the amebas, it seems logical to assume that they may be a source of a growth factor or factors; for example, when Jacobs (53, 54) cultivated the amebas in the presence of *heat killed* bacteria, the dead bacteria must have furnished some growth promoting factor. Interestingly, Jacobs (53) found, however, that dead yeast, yeast juices, *ether killed* bacteria, and plasmoptyzates of bacteria had no growth promoting activity on the amebas. Shaffer *et al.* (92, 93, 94) maintained cultures of amebas in the absence of actively multiplying bacteria although bacterial sterility was not established. As a means of supplying the necessary growth factors to the culture tube, they added a bacteria-free filtrate from a culture of four species of bacteria growing together (94). Here, at least, the factors were in the filtrate rather than in the cells themselves. Karlsson (58) reported on a growth stimulatory factor in dead bacterial cells which was labile to heat, to alkali, and to oxidation, but stable in the presence of formaldehyde.

Ovahormone, hemoglobin, horse red blood cells, and vitamins of the B complex added to Dobell and Laidlaw's medium had a stimulatory action on amebic growth (107). DeLamater and Hallman (29) detected a heat-stable, dialyzable

substance from the protein-free fraction of human serum which they believed to be essential to the growth of a strain of *E. histolytica*. Rees *et al.* (14, 87) thought that some component in egg white contributing to the growth of *E. histolytica* may be rendered inactive by oxidation. When the medium containing egg white was placed in a cotton stoppered tube and stored, the medium lost its ability to support the amebas, whereas the same medium in a rubber stoppered tube in which the residual oxygen was absorbed by pyrogallic acid showed no loss of ameba maintaining properties. Hitchcock and Rawson (49) suggested that some substance (or substances) in fresh and dehydrated coagulated egg yolk was a nutrient factor for *E. histolytica*. This substance was soluble in saline and thermostable. Nelson (69) stated that when egg yolk was used in a medium, an agar base was necessary because the egg yolk suspended in saline alone yielded an abundance of lipid globules, especially lecithin globules, which were ingested by the amebas and forestalled starch ingestion essential to the growth of the organisms. This worker (70) also implied that small amounts of agar were necessary for growth of the amebas; however, highly purified agar was incapable of supporting growth unless magnesium in the form of sulfate, chloride, or acetate was added.

The role of cholesterol was first presented by Snyder and Meleney (96), who stated that it was required for the growth of *E. histolytica*. Following this, Rees *et al.* (86) found cholesterol and 8 vitamins of the B complex stimulated action on the growth of amebas in media containing egg white. Cholesterol alone, or the vitamins alone, in egg white medium had no effect, but in combination the stimulation was pronounced. Amino acids, purine bases, ovalbumin, ovomucin, and ovomucoid were added to the medium with only slight increases in ameba numbers. Cholesterol has been used in several culture media (45, 46). Hansen and Anderson (46) described a medium containing amino acids, vitamins of the B complex, nucleic acid, cholesterol, and rice starch. It is probable that these growth substances furnish nutritional factors for the associated organism *t*, and in this way indirectly affect the amebic populations. Griffin and McCarten (39) reported that cholesterol in adequate amounts could be used as a substitute for serum in culture media and that oleic acid in optimum concentrations reinforced the action of cholesterol.

Karlsson and Nakamura (59) found a growth stimulatory factor for *E. histolytica* in fresh yeast cake; this factor was highly unstable and was lost upon Seitz filtration. Although some workers (31) found that fecal particles or fecal extracts added to culture media had no effect on the amebas, Andrews, Johnson, and Schwartz (2) reported that the growth of amebas was increased by the addition of extracts of human feces to the culture fluid. This extract was water soluble, passed through the Berkefeld filter, and was inactivated by heating at 60 C for 30 minutes.

Although various substances have been reported as growth factors or growth stimulants for *E. histolytica*, much more work remains to be done on pure cultures of the amebas before the factors can be established as being definitely required by these organisms. The presence of an associated organism in cultures

of *E. histolytica*, complicates the nutritional studies and even the physiological studies. When the associated organism flourishes, the amebas also grow well; when the associated organism grows poorly, the amebas also fail. With such an association, it is extremely difficult to determine whether we are introducing a factor that stimulates the amebas directly or stimulates the bacteria, and thus has an indirect stimulatory effect on the amebas. Another point to consider is that an increased count of amebas usually has been reported as stimulation without taking into account the normal cyclic fluctuations in amebic populations, and also without standardized procedures. Any claim for a growth factor for *E. histolytica* must be accepted with due regard for the foregoing complications.

III. GROWTH BEHAVIOR AND MULTIPLICATION RATES

Boeck and Drbohlav (9) reported that the amebas grew mainly at the bottom of the culture tubes in the bacterial sediment. Stained microscopic sections showed that the organisms did not penetrate into the slant. Migration of *E. histolytica* over solid media was noted by Snyder and Meleney (97). In test tube cultures, the amebas were confined to the bottom 0.25 ml of the media (1); two factors contributed to this result: the specific gravity of the amebas and the oxidation-reduction potential of the medium. The number of amebas varied but was greatest on the second day after inoculation, with the average life of cultures being 4 to 5 days (9). The usual procedure at the present time is to transfer cultures of *E. histolytica* every 48 hours. In cultures of amebas growing with *T. cruzi*, the amebas settle to the bottom, but many are seen on the sides of the test tubes throughout the length of the tubes (personal observation). Balamuth and Howard (6) stimulated amebic growth by frequent agitation of the medium; they postulated that the amebas grew in local "nests" at the bottom of the culture tubes and that stirring broke up these clumps, scattering individuals over the substratum. They thought that in this way more effective use was made of the available bottom surface.

Chinn *et al.* (20) stated that the initial inoculum of amebas had to be great enough to overcome the lag phase of growth, during which time there was a high mortality among the amebas. Inocula containing from 1,000 to 100,000 amebas in approximately 0.5 ml of the parent culture showed no lag phase (1). As few as 500 amebas in association with organism *t* resulted in positive cultures according to Brackett and Bliznick (11), but occasionally as few as 15 amebas gave positive results. The terminal population of amebas was independent of the number of amebas in the inoculum. These workers showed a maximum count in ameba populations in a single transfer to be 36,000 or about fifteen generations. Anderson and Hansen (1) found the maximum number of amebas in the average test tube containing 5 ml of medium to be about five hundred thousand. The average number of divisions per ameba per day was from two to four (1, 11).

Tracing the growth of *E. histolytica* in the culture media of Cleveland and Collier (22), Dobell and Laidlaw (31), and Balamuth and Sandza (5), Balamuth

and Howard (6) learned that the growth curves of amebas and bacteria followed the classical bacterial pattern although higher yields occurred in the richer media. They concluded that the lag phase of amebic growth was relatively prolonged in comparison with bacterial growth, although the lag phase was shortened in media pre-conditioned by growth of bacteria alone. Populations of bacteria and amebas were plotted in the form of growth curves for six bacterial types in a monobacterial culture, together with the relative growth response of the amebas. No correlation was observed between the relative ranking of bacterial and amebic growth (7). Similar studies by Faust *et al.* (34) showed that amebic population growth curves approximated those reported for typical bacterial growths. Cultures of *Endamoeba terrapinae* showed cyclic alterations of vitality with periods of approximately 90, 180, and 360 days. The periodicity was associated with total age of culture rather than with frequency of transfer (42). An increase in the bottom surface area of the culture vessel enhanced the population of the amebas, whereas additional vertical surface had no effect (34).

According to Griffin and McCarten (40), the major sources of variation in cultures of entozoic amebas are associated with age of culture and quantity of starch. They suggested that this variation is associated with inconsistent behavior of the cultures used for inoculation. To eliminary variability, the following factors should be provided: Standard media, constant physical environment, a single pool to inoculate all cultures, constant quantity of starch, constant size of inoculum, and constant age of culture (41).

Two races of *E. histolytica* have been reported (91, 98). The small race had trophozoites with a mean size of 7.17 microns and the large race had trophozoites of a mean size of 12.85 microns. It was exceedingly difficult to cultivate the smaller race.

IV. PHYSICOCHEMICAL FACTORS

The various physicochemical factors that enter into the physiology of an organism appear to be especially critical for *E. histolytica*. Inconsistent results have been reported in the study of the various effects of physical and chemical factors, but this is not surprising when one considers that experiments were performed on the amebas growing in association with different bacterial species.

1. *pH factor.* Little work has been done on the role of pH in the growth of *E. histolytica.* Boeck and Drbohlav (9) reported that the amebas grew best in cultures having an initial pH of 7.2 to 7.8. After 24 hours of incubation, all tubes had a pH of 6.0 or lower because of the fermentative activity of the bacteria. These experiments were confirmed by Craig (27) who stated that cultures with an initial pH of 6.0 had no living amebas after 48 hours. Chang (16) thought that the maintenance of a favorable pH range throughout the period of incubation depended not only on the initial pH value but also on the accompanying bacterial flora.

2. *Oxidation-reduction potential.* Jacobs (52) found that growth of amebas occurred at an oxidation-reduction potential of -114 to -150 mv, but growth was more prolific at -300 to -500 mv. The longevity of the ameba cultures

was correlated with the maintenance of reducing potentials. He believed that the cultivation of *E. histolytica* without bacteria would require control of the oxidation-reduction potentials of the medium. However, in a later communication, Jacobs (55) reported that *E. histolytica* can grow and reproduce within a wide range of oxidation-reduction potentials. He found no correlation between the maintenance of low O-R potentials and the growth of amebas although much better growth of amebas occurred in the presence of bacteria which produced low potentials as compared with bacteria which did not. A possible role of the bacterial flora growing in association with the amebas was to maintain a suitable reducing potential (18). Chang (18) found a close relationship between the O-R potential in the culture medium and the growth of *E. histolytica*. Best growth was observed at potentials between −350 and −425 mv, less at potentials between −275 and −350 mv, and still less between −200 and −275 mv. Between potentials of −50 and −150 mv no increase in numbers occurred, while the trophozoites were found dead at potentials of −50 to +50 mv. According to Hopkins and Warner (50), the optimum O-R potential for amebic growth was well below −400 mv, and a redox potential above the optimum caused destructive oxidation, while below that potential normal oxidation of foodstuffs was prevented. In cultures of the amebas grown with *T. cruzi*, the maintenance of a low potential was not too critical (64).

To maintain a reduced potential, investigators have used many chemical agents in culture media. Hansen (45) used cysteine; others have used thioglycolate (40, 41, 64, 94), and Pautrizel *et al.* (71) found that even in the presence of bacteria, reducing substances such as reductose, which is rich in reductone, decidedly favored growth of amebas. The addition of sodium hydrosulfite, a reducing agent, in small quantities to ameba preparations caused the formation of large clear vacuoles in the amebas (50); the addition of excessive amounts of reducing agents stopped all activities and the amebas died. Exclusion of atmospheric oxygen by the use of a petrolatum seal has been used as a means of maintaining reducing potentials (12, 95).

3. *Anaerobiosis.* Earlier reports by Dobell and Laidlaw (31) stated that *E. histolytica* grew under aerobic conditions, and it was not until Snyder and Meleney's (96) publication that anaerobic conditions were considered essential for the growth of the amebas. Their evidence indicated that marked reduction of the oxygen tension and probably elevation of the carbon dioxide tension were essential for growth (97). Hartman (48) found that the amebas grew satisfactorily with a carbon dioxide tension up to 50%.

Many workers have concluded that the presence of free oxygen was definitely harmful to *E. histolytica* (6, 12, 50, 95). Shaffer *et al.* (95) were not sure whether the oxygen was directly harmful to the amebas or whether oxidation destroyed some essential O_2-labile factor needed by the amebas. As already mentioned, a petrolatum seal has been used to eliminate atmospheric oxygen (12, 95), but Nakamura (64) showed that amebas in association with *T. cruzi* grew abundantly even though the sodium thioglycolate and petrolatum seal, used in the standard procedure, were eliminated. Rees *et al.* (14, 87) learned that cotton

stoppered media which had become oxygenated upon storage were less favorable for growth of amebas than the same media stored with an anaerobic seal to prevent atmospheric oxygen from entering. Jacobs (55) thought that there was not enough evidence to conclude that complete removal of oxygen was necessary for growth of *E. histolytica*. He postulated that it was possible for the amebas to grow and multiply in an environment containing small amounts of oxygen if an adequate substrate was present. Information currently available indicates that anaerobic conditions are necessary for optimum growth and metabolism.

4. *Effect of temperature.* The optimum temperature for the growth of *E. histolytica* is between 37 and 38 C (9, 31). Cysts of the amebas survived for 30 minutes at 45 C but did not survive for 5 minutes at 50 C (108). Jones and Newton (57) exposed cysts to temperatures of 45 to 54.5 C and found that at 45 C the time required for complete cyst destruction was more than 2 hours. At temperatures of 46 to 47 C all cysts were destroyed within 1 hour, and at a temperature of 48 C cyst destruction was completed in 30 minutes. At 49 to 50 C complete cyst destruction was effected within 10 minutes; at 51 C all cysts were destroyed within 5 minutes; and at 52 to 54.5 C all cysts were destroyed within 1 minute. Chang (19) made a quantitative analysis of the data on the destruction of cysts. The data showed a semilogarithmic relationship between the increase in the most probable cysticidal contact times and the lowering of the temperatures at which the cysts were exposed. Analysis of this relationship showed that the value of the energy of activation for the thermodestruction of cysts was 134,000 calories.

Results of Nakamura and Anderson (65) indicated that *E. histolytica* trophozoites survived heat treatment at 45 C, 48 C, and 50 C for 10 minutes. Survival time of the trophozoites ranged from 48 to 96 hours at refrigeration temperature; 6 to 16 hours at room temperature; and 2 to 5 hours at an incubation temperature of 37 C (106). Another set of data showed that cysts kept at 4 to 5 C were viable for 40 days (17). Discrepancies in the viable period of either trophozoites or cysts might be explained by the differences in the number of cysts and the difference in the percentage of mature cysts present in the tests (17). Cysts of *E. histolytica* dried in air at 80 C were damaged irreversibly; on the other hand, *Endamoeba coli* treated similarly produced cultures upon subculturing (77, 78).

5. *Effect of other factors. E. histolytica* can tolerate considerable changes in tonicity (31). A medium with a salt content of 0.94% was shown to be optimal (31). According to Chang (16) the optimum salt concentration in the medium was M/30 phosphate and 0.4% sodium chloride. Nelson (70) thought that the phosphate buffer was essential; growth decreased when the phosphate buffer was reduced below M/40, and at M/80 dilution growth failed.

Ameba cultures centrifuged for 15 minutes at speeds of 1,500, 2,000, 2,500, 5,000, 8,000, and 12,500 rpm were not significantly destroyed (106a). Repetitious centrifugation of the amebas at 1,000 to 1,500 rpm had no effect on the amebas (61, 67).

Sadun *et al.* (88) showed that a single irradiation exposure of the amebas to 30,000 roentgen units did not affect their growth. Exposures of 60,000 and 120,000 r inhibited the ameba population although viable amebas were observed for 16 transfers after irradiation with 120,000 r.

V. BACTERIAL ASSOCIATES

1. *Role of the bacterial associate.* Except for temporarily maintained cultures where bacteria were apparently absent or multiplying only slowly (54, 63, 92, 93, 94, 95), *E. histolytica* has not been cultivated free of an associated organism. The advantages of obtaining a pure culture of *E. histolytica* are obvious. Until 1950, when Phillips and Rees (73, 75) cultivated the amebas in association with *T. cruzi*, the amebas had never been cultivated without bacteria. It became accepted that bacteria were absolutely essential for the cultivation of the amebas for, in spite of repeated attempts, a pure culture of *E. histolytica* was not achieved. Observing such an interesting dependent association where the amebas cannot survive when the bacteria are removed or killed, the role of the associate bacteria becomes a challenging problem.

Just what do the bacteria furnish the amebas in culture? One concept is that the bacterial flora may provide suitable anaerobic conditions or reduced O-R potentials (6, 12, 18, 50, 64, 95, 96). However, if this were the sole requirement which the bacteria fulfilled, certainly a number of reducing agents are available which could be added to the medium, or the atmospheric oxygen could be excluded from the medium. A second concept is that the products of bacterial metabolism are used by the amebas (6, 18, 64). Rees *et al.* (84) postulated that the bacteria attacked food materials producing hydrolytic products in the test tube that were similar to those produced in the intestinal tract or in other tracts parasitized by both bacteria and protozoa. This idea that bacterial metabolic products are used by the amebas has been widely accepted. According to most workers, the decline of bacterial populations affects the amebas adversely; however, Spingarn and Edelman (99) prolonged the survival of the trophozoites by killing the bacteria with streptomycin. Their hypothesis was that the rapid disappearance of amebas in culture media was due to bacterial overgrowth which produced an environment unfavorable for the survival of the amebas. However, since *E. histolytica* develops in the liver in the absence of bacteria, it seems logical that metabolic products of the bacteria can be replaced by factors found in the liver. A third concept is that the bacteria themselves may provide food for the amebas (84). This, too, is a possibility because cultures of the amebas have been maintained on heat killed bacteria (54) and growth factors for *E. histolytica* have been found in dead bacterial cells (55, 58). However, this type of cultivation is not always possible, and many workers have attempted it without success (21). A fourth concept is that the bacterial flora may provide enzymic systems which the amebas require to utilize food material in the medium (18, 64). This has been recently studied in the form of a culture medium which substituted an enzyme preparation for the associated organism (63).

At the present time, the use of complex media, monobacterial associates of

different species, and multiple bacterial associates make it difficult to elaborate the exact role of the bacteria in connection with the growth of *E. histolytica*. A comparative biochemical study of the bacteria that support amebic growth would be of a definite value in studying their role. It would be interesting to learn what is found in common among the bacteria that have been shown to support the amebas; possibly enzyme systems or metabolic products common to all supporting bacteria are furnished by them to the amebas.

2. *Elimination of the bacteria.* To study the physiology and metabolism of the amebas, many attempts have been made to eliminate the bacteria from the amebas. Furthermore, elimination of bacteria would provide an inoculum of amebas freed of associates which can be used to test new media showing promise of sustaining amebic growth without bacteria. Gemar (38) attempted to reduce bacteria from ameba cultures by inhibiting the bacteria with bacteriophage. As only a transitory inhibition of bacteria occurred, this method was unsuccessful in ridding the amebas of bacteria. The bacteriophage did not affect the amebas in any way. Many cultures of *E. histolytica* containing *Blastocystis hominis* have eliminated the latter by 1:50,000 dilution of neutral acriflavine (101). Rees (80, 81) constructed a micromanipulator for isolating protozoa. Using this manipulating micropipette under the compound microscope, he isolated individual trophozoites of *Trichomonas foetus* from the vaginal fluid of a cow (80). These trophozoites were washed free of bacteria and inoculated into culture media to secure a bacteria-free pure culture of *T. foetus*. Rees *et al.* (83) applied the same technique with hopes of establishing a bacteria-free pure culture of *E. histolytica* but were not successful. They obtained amebas freed of bacteria, but cultures could not be established unless bacteria were added. They have reviewed the problems encountered in the growth of *E. histolytica* in cultures developed by microisolation (83).

Since the advent of antibiotics, penicillin has been used to eliminate the bacteria from ameba cultures (53, 54, 67, 92, 93, 94). Balamuth and Wieboldt (7, 8) stated that the use of 1:50,000 mercuric chloride for 90 minutes at 4 C was more reliable and more convenient than the use of a micromanipulator. Rao (76) used a combination of N/20 hydrochloric acid, 0.002% mercuric chloride, and 0.02% potassium permanganate which killed the bacteria present in amebic cultures. In the concentrations used, these agents had no adverse effects upon the amebas. A combination of several methods was used by Miller and Firlotte (61); centrifugation and levigation were followed by passage of amebas, by micromanipulation, through a series of solutions containing penicillin and streptomycin. Others have employed similar techniques (67). Shaffer and Frye (92) used heat, penicillin, and streptomycin. The use of heat to eliminate bacteria is not very satisfactory since temperatures necessary to inactivate the bacteria are detrimental to the amebas. In this connection, Phillips (73) stated that one of the advantages in maintaining an *E. histolytica-T. cruzi* culture is that trypanosomes can be eliminated by inactivation at temperatures slightly higher than 37 C, hence, without much destructive effects on proteins and enzyme systems as compared with the heat killing of bacteria.

The methods available for eliminating bacteria from ameba cultures do not insure absolute sterility. It is extremely difficult to obtain a 100% kill of bacteria with antibiotics used in a range that is not toxic to the amebas. If it is desired to kill most of the associated organisms so that growth can be studied in an environment relatively free of these organisms, these methods are satisfactory. However, if a pure culture, completely devoid of other organisms, is desired, these methods are crude and unsatisfactory. A method is yet to be developed that will consistently eliminate completely associated organisms with little damage to the amebas.

3. *Monobacterial cultures.* The purposes of establishing a monobacterial associate of *E. histolytica* are several fold. For example, amebas growing in association with a multiple flora, where the fluctuations in the bacterial populations are uncontrollable, may furnish misleading results. A monobacterial culture, at least, will eliminate much of the variability. This type of culture furnishes a better tool for physiologic and metabolic studies of the amebas and is the next best thing to the amebas in pure culture form. At present, monobacterial cultures of *E. histolytica* are maintained in many laboratories throughout the world. One of the main functions of eliminating bacteria from ameba cultures, whether by mechanical or chemical means, was to introduce a single known bacterium. If the metabolism of the bacterium introduced is known, a comparative study may reveal the activities of the amebas.

Rees *et al.* (85) established the first monobacterial culture of *E. histolytica.* The associate bacterium was designated organism *t*, which was a microaerophilic, rod shaped bacterium. Many bacteria have been used as the single associate organism in cultivating the amebas. Chinn *et al.* (20) studied 26 species of bacteria with regard to their ability to maintain *in vitro* growth of amebas. A heterogeneous group of 14 species was found capable of supporting growth. Some of the intestinal bacteria such as *Alcaligenes faecalis* could not initiate growth of amebas, and three species of yeasts also failed. Additional bacterial species which will maintain amebas in a monobacterial culture have been reported (7, 8, 56, 90). However, amebas with a mixed bacterial flora grew better than with monobacterial associates (7, 20).

Luttermoser and Phillips (60) transferred trophozoites from *E. histolytica* growing with a single bacterium to a culture of *T. cruzi*, establishing an ameba-trypanosome culture. Nakamura (62) attempted to exchange associated organisms in cultures of *E. histolytica-T. cruzi* with an ameba-*t* culture and with an ameba-*Streptobacillus* sp. culture without success. Ability to exchange associated organisms is useful in obtaining the desired combination when wanted. Also one can initiate a new monobacterial culture at will, providing the previous associate is eliminated completely before the amebas are transferred to the new associate.

4. *Bacteria-free cultures.* Cleveland and Sanders (21) were the first to obtain amebas without bacteria. They produced amebic abscesses by injecting ameba cultures directly into the liver of a cat with a hypodermic needle following laparotomy. The bacteria were destroyed by the liver, and 7 days later the amebas

were present in a bacteria-free amebic abscess. These abscesses were removed from the liver with sterile instruments. After removing the outer surface of the abscess, the material containing the amebas was inoculated into various culture media. Since none of the amebas survived without the addition of bacteria, this was not a bacteria-free culture, but it was the initial step towards one. In fact, this procedure still remains one of the most satisfactory methods of obtaining amebas free of bacteria. Friedrichs and Harris (35) removed material from the hepatic lesions of a man. Sterility tests of the abscesses indicated an absence of bacteria, but by the time culture media were inoculated contamination had occurred.

The apparently bacteria-free cultures of Jacobs (53, 54) have already been mentioned. A stock culture was maintained for as long as 6 months without any evidence of bacteria, but growth was quite limited. The cultures of Shaffer *et al.* (92, 93, 94) have also been mentioned. With the aid of antibiotics they maintained a culture of amebas for 100 transplants without any appreciable growth of bacteria.

5. *Monoprotozoal cultures.* Phillips and Rees (73, 75) initiated the monoprotozoal associate culture of *E. histolytica* with *T. cruzi*, and the amebas grew well whether the trypanosomes were alive or killed by exposure to a temperature of 48 C for 10 minutes. Rich suspensions of the trypanosomes were necessary for good growth of *E. histolytica*. The growth of amebas was excellent when the count of trypanosomes was 50 million per ml, but when the count was one million per ml, multiplication did not occur (75). Other species of Trypanosomidae were studied (74); maximum growth occurred with *Strigomonas fasciculata*, but little growth occurred with *Trypanosoma conorhini*, *Leishmania donovani*, *L. tropica*, and *L. brasiliensis*. The amebas could not be cultivated in association with *Trichomonas foetus* (82). It would be extremely interesting to learn if other pathogenic trypanosomes can support amebic growth. Another approach to the cultivation of *E. histolytica* might be to compare the various Trypanosomidae to see what differences exist to account for the fact that *T. cruzi* supports growth of amebas while the other Trypanosomidae do not.

VI. METABOLISM

Metabolic studies of significant importance cannot be performed in the absence of a pure culture. Realizing that one of the key approaches to chemotherapy is through the metabolism of the parasite, numerous attempts have been made to study the metabolic pattern of *E. histolytica*. Thus far, the results have been discouraging.

Experiments performed on cultures of amebas growing with organism *t* demonstrated that gas was produced from glucose in equal amount and similar composition by cultures of the bacterium alone and cultures of the amebas plus the bacterium (13). The predominant constituent of the gases produced was hydrogen, suggesting an anaerobic metabolism. Other gases found were carbon di-

oxide, methane, and nitrogen (probably residual). Even if different amounts of gas were produced by ameba-organism *t* as compared with organism *t* alone, the results could be misleading because of the possibility of a synergistic relationship.

Carrera (15) concluded that *E. histolytica* has a high concentration of acid phosphatase on the basis of intense acid phosphatase activity in the areas of amebic invasion in experimental animals.

Manometric measurements of acid production by the amebas were recorded by Nakamura *et al.* (66, 67). Penicillin and streptomycin were added to the cultures to kill the associated bacteria. The amebas were pooled, washed, and centrifuged; the activity of the supernatant was taken due to the bacteria alone if any were present. The concentrated amebas numbered 2,000,000 per ml. Buffer, additional antibiotics, cysteine, glucose, and amebas were placed in the Warburg vessels, which were cleared of air with a carbon dioxide-nitrogen mixture, and manometric measurements were taken over a 70 minute period. These studies demonstrate metabolic acid production by *E. histolytica*. Further investigations should be concerned with both qualitative and quantitative aspects of this acid production. The metabolic studies of *E. histolytica* have been applied as a new technique in the evaluation of potential amebacidal agents. As seen in table 1, the inhibitory effects of the amebacides on the manometric metabolism of the amebas may be of value in studying the action of these drugs (67).

VII. CONCLUSIONS

In summary, it is clear that the nutritional requirements of *Endamoeba histolytica* have not yet been established. Although various types of culture media are available, all are complex, and a synthetic medium for the cultivation of the amebas must await a pure culture of this organism. Preparing a synthetic medium for the amebas in association with another organism will not elucidate the nutritional status of the amebas since it cannot be determined whether the medium provides the nutritional basis for *E. histolytica* or for the associated organisms. The primary task confronting us then is a pure culture of the amebas. At this stage it does not really matter whether the medium is complex or simple, providing associated forms of life can be completely eliminated. Once a pure culture is established, growth factors can be eventually determined.

Many growth factors and growth stimulants for *E. histolytica* have been reported in the literature, but evaluation of the claims is difficult. Some substance, increasing the population of the amebas, immediately is labeled a growth factor or growth stimulant without learning whether the bacterial population remained constant or whether it too increased in numbers. Ordinarily, substances which stimulate growth of bacteria simultaneously will indirectly stimulate amebic growth. An assay technique developed by Karlsson (58, 59) measures increase in ameba numbers in an environment where the bacteria are either dead or dying. This type of assay for growth factors may be of value, assuming the bacteria are not contributing to the increase in ameba populations.

To determine the nutritional requirements of *E. histolytica* it will be necessary to study each component of the media to learn what it furnishes the amebas. Liver preparations have been shown to be satisfactory for the cultivation of the amebas. Just what does liver supply in these media? Many workers think that vitamins, amino acids, and purine bases are supplied, whereas others believe that some unspecified type of compound is present in liver which the ameba can use for metabolism. Living bacteria that support growth of the amebas will not always support growth when killed or inhibited. Are actively metabo-

TABLE 1

Effect of drugs on the metabolic activity of ameba

DRUG	CONCENTRATION	GAS FORMATION*	
		Range†	Mean
Fumagillin	1/20,000	81.3–97.4	90.8
Terramycin	1/20,000	6.6–16.7	11.7
Aureomycin	1/20,000	82.4–88.0	85.4
Emetine	1/20,000	20.2–71.0	56.0
Thiocarbarsone	1/20,000	22.3–39.5	32.1
Fumagillin	1/80,000	0.0–11.7	4.6
Terramycin	1/80,000	7.3–20.8	14.2
Aureomycin	1/80,000	45.6–71.1	56.8
Fumagillin +	1/80,000		
Thoicarbarsone	1/20,000	53.5–87.0	61.6‡
Fumagillin +	1/80,000		
Terramycin	1/20,000	92.2–100.0	97.2‡
Fumagillin +	1/20,000		
Aureomycin	1/20,000	86.5–100.0	97.3
Aureomycin +	1/80,000		
Terramycin	1/80,000	65.3–88.0	72.6
Thiocarbarsone +	1/20,000		
Emetine	1/20,000	73.8–92.5	82.3

* Acid + CO_2 during 70 minute period expressed as per cent inhibition of the control without drug.

† Five experiments.

‡ Statistical evidence of synergism was obtained for these two combinations only. *i.e.*, more than an additive effect.

lizing bacteria necessary for the growth of the amebas? Or perhaps, is some factor found in the bacterial cell which is stimulatory when ingested by the amebas? Experimental results indicate that an affirmative answer may be given to both questions. It is possible that the material furnished by actively multiplying bacteria is also furnished by killed bacterial cells but not in high enough concentrations to maintain the growth of the amebas. Also, we must not overlook the possibility that the bacterial metabolism maintains a low oxidation-reduction potential which is optimal for the growth of *E. histolytica*. However, many anaerobic bacteria, which do not maintain such reducing potentials, have

been used for the cultivation of the amebas. One approach which has not been studied is the use of sterile cells of *E. histolytica*, both intact and lysed, as growth promoters for the amebas in cultures. These cells may be prepared by growing the amebas in the conventional manner and then separating them from the bacteria by differential centrifugation.

Much remains to be done on the possibility of incorporating enzymes into the media as substitutes for bacteria. It seems probable that the amebas lack many enzyme systems which are a part of the associated bacteria. If certain metabolic reactions were necessary for growth which are accomplished by the enzymes present in the bacteria, it may suffice to furnish these enzyme systems to the ameba cultures to replace the need for the associated bacteria. However, the multitude of enzymes a bacterium possesses makes this type of study difficult.

Another approach to the physiology of *E. histolytica* which has remained unexplored is the determination of the enzyme systems the amebas possess. If the enzymatic makeup is determined, it will be easier to predict the metabolic pathways of the various foodstuffs available in the media. After the amebas are concentrated, washed, and freed of bacteria, the enzyme systems present can be studied by histochemical and manometric methods.

Practically nothing is known of the metabolism of the amebas. Studies with manometric techniques have shown that small amounts of acid are produced with glucose as the substrate. Further study should be directed towards determining the nature of the acids produced and also whether or not other carbohydrates can be utilized as an energy source. It would also be interesting to learn if acid production will occur with rice starch as the sole substrate—and something about the metabolism of amino acids.

During the past few years much effort has been directed toward the study of the physiology and metabolism of *E. histolytica*. When extension of these studies eventually reveals the metabolic pattern of the amebas, a more rational therapy will become available against amebiasis.

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